SYMPOSIUM

Female Reproductive Fluid Increases the Opportunities for Postmating Sexual Selection by Prolonging the Egg Fertilization Window*

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ABSTRACT: Female reproductive fluid (the fluid that surrounds the eggs) has attracted increasing attention for its role in fertilization and postmating sexual selection through its effects on sperm traits. Surprisingly, however, only a few studies have investigated the effects of the female reproductive fluid on the eggs. Yet these effects might offer great potential to affect fertilization dynamics by, for example, increasing the opportunities for postmating sexual selection. Here, we determined whether the female reproductive fluid, by extending the egg fertilization window (the time available for egg fertilization), could also increase the opportunities for multiple paternity. Using the zebrafish (*Danio rerio*), we first tested the prediction that female reproductive fluid increases the egg fertilization window; then, using a split-brood design with the sperm of two males added at different time points after egg activation, we tested whether the degree of multiple paternity varies in the presence or absence of female reproductive fluid. Our results reveal the potential of female reproductive fluid to increase multiple paternity through its effects on the egg fertilization window, thus broadening our knowledge of how female mechanisms affect postmating sexual selection in externally fertilizing species.

Keywords: sexual selection, sperm competition, female reproductive fluid, ovarian fluid, zebrafish, egg viability.

Introduction

When females mate with multiple males within the same reproductive episode, sexual selection can continue after mating in the form of postmating sexual selection (Birkhead and Pizzari 2002). Traditionally, studies of postmating sexual selection have focused mainly on the interplay among gametes (i.e., sperm and eggs). However, more recently it has also been demonstrated that the nongametic components released with sperm and eggs play an important role in this stage of sexual selection. Decades of studies on the fluid that surrounds the sperm—namely, the seminal fluid—have revealed that it has a multitude of effects on different aspects of the fertilization processes that affect postmating sexual selection (reviewed in Perry et al. 2013). For example, seminal fluid can deeply affect female remating behavior and the outcome of sperm competition (i.e., the competition of sperm from two or more males to fertilize the same batch of eggs; Parker 1970) by affecting sperm competitiveness (Poiani 2006; Ramm 2020).

Interestingly, however, in this context, the counterpart of seminal fluid for females seems to have been overlooked. This fluid, recently named (to avoid specific taxa-related terminology) "female reproductive fluid" (FRF; Gasparini et al. 2020), is a fluid that can have different origins (ovarian, oviductal, follicular, and/or coelomic, hence explaining some of the different terminology used in the literature, such as ovarian fluid, spermathecal fluid, gonoductal fluid, egg water, follicular fluid, etc.) but has the common denominator of surrounding the eggs before and at the time of fertilization. This fluid is kept inside the female reproductive tract in internal fertilizers or is released along with

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the eggs in external fertilizers, and ultimately it is the fluid the sperm come into contact with during their quest to fertilize the eggs (Zadmajid et al. 2019; Gasparini et al. 2020).

FRF has been shown, across a variety of internally and externally fertilizing taxa, to affect multiple sperm traits important for fertilization success, generally enhancing spermatozoa performance. For instance, FRF mediates sperm attraction, prolongs the duration of sperm motility, modulates sperm trajectory, increases sperm viability, and enhances sperm velocity and sperm motility (e.g., Oliveira et al. 1999; Bernasconi et al. 2002; Urbach et al. 2005; Elofsson et al. 2006; Rosengrave et al. 2009; Gasparini et al. 2012; Gasparini and Evans 2013; Alonzo et al. 2016; Liberti et al. 2016; Poli et al. 2019; Myers et al. 2020).

Interestingly, in recent years accumulating evidence has revealed that the effects of FRF on sperm traits can extend into postmating sexual selection, with evidence of FRF as a mediator of cryptic female choice, able to differentially affect sperm of different males and ultimately bias the outcome of sperm competition (Firman et al. 2017; Gasparini et al. 2020). For example, FRF mediates sperm selection to avoid inbreeding by favoring unrelated males during fertilization in the guppy (*Poecilia reticulata*; Gasparini and Pilastro 2011) and the chinook salmon (*Oncorhyinchus tshawytscha*; Lehnert et al. 2017), while in the external fertilizer mussel (*Mytilus galloprovincialis*), FRF has been shown to attract the sperm of the more genetically compatible males (Oliver and Evans 2014). Also, FRF has been shown to mediate sperm selection toward the preferred male phenotype, and this seems to occur in species where males show alternative mating tactics, as in the ocellated wrasse (*Symphodus ocellatus*), where FRF decreases the relative importance of sperm number over sperm velocity, thereby penalizing the numerical advantage of sneaker males (Alonzo et al. 2016). Despite this growing body of evidence indicating the role of FRF in postmating sexual selection processes through its effects on sperm traits, not many studies have investigated the effects of FRF on eggs, and none so far have explored these effects from the perspective of postmating sexual selection.

The primary role of FRF is to provide the appropriate environment for oocyte maturation, fertilization, and early embryo development (Leese et al. 2001; Aguilar and Reyley 2005). FRF prolongs egg life span in external fertilizers (Dietrich et al. 2012) and improves eggs quality, as it is involved in the protection of eggs from oxidative stress (Agarwal et al. 2005; Da Broi et al. 2018) and pathogens (Johnson et al. 2014). Enzymes of antioxidant defense have been found in the FRF of various species from insects (Baer et al. 2009) to mammals (Harvey et al. 1995; Fu et al. 2016), and proteomic studies have revealed the presence of proteins related to the immune system in both internally and externally fertilizing species (Seppola et al. 2009; Zamah et al. 2015; Dosselli et al. 2019). Despite this evidence of the effects of FRF on eggs, the consequences of those effects for postmating sexual selection have yet to be explored.

Here, using the zebrafish (*Danio rerio*), we test for a potential role of FRF in sexual section processes mediated by the effects on eggs rather than on sperm. We asked whether the FRF, by affecting egg viability, might also extend the fertilization window of the eggs (i.e., the time window available for eggs' fertilization) and whether this can translate into increased opportunities for postmating sexual selection. Indeed, theoretical models (Harts and Kokko 2013) suggest that the length of the fertilization window might be an important factor that is able to shift the balance between pre- and postmating sexual selection, with a wider fertilization window associated with the increased importance of postmating mechanisms of sexual selection. The zebrafish is well suited to test this hypothesis for many reasons. Zebrafish are group spawners and egg scatterers, and in the wild females dart repeatedly into shallow water (1–2 cm deep) when ready to spawn, often chased by multiple males (Engeszer et al. 2007; Spence et al. 2008), frequently resulting in broods with multiple paternity (Watt et al. 2011). Once released by the female, eggs are activated by contact with freshwater but within 1 min become nonfertilizable (Yamamoto 1961). Recent findings have shown the potential for FRF to affect postmating dynamics in this species based on the effects of FRF on sperm traits (Poli et al. 2019), thus also suggesting the possibility for FRF to have other effects on the fertilization process.

We first determined the duration of the egg fertilization window and assessed whether the presence of FRF can affect it. Then, using a split-brood design with sperm from two males added at different time points from egg activation, we tested the prediction that the presence of FRF can increase the opportunities for multiple paternity, suggesting a novel mechanism of FRF to influence postmating sexual selection.

Material and Methods

Fish Maintenance

Zebrafish used in this experiment were Tübingen wild type, reared under standard laboratory conditions at the Zebrafish Facility of the Department of Biology, University of Padova, Italy. Adult males and females were kept separated in groups of 15 fish in 3-L tanks in a recirculating rack system (Tecniplast) at a water temperature of 28°C ± 1°C with a 12L : 12D photoperiod. All fish were fed ad lib. three times per day with a mix of dry food and *Artemia* nauplii. Both males and females used for the experiments were 7–9 months old. All experiments were performed in

accordance with the relevant Italian and European legislation and were approved by the Ethics Committee of the University of Padova (approval 100/2019).

Experimental Design

Experiment A: Estimating the Zebrafish Fertilization Window. We conducted a preliminary experiment to estimate the egg fertilization window in zebrafish under standard conditions (i.e., with no manipulation of the FRF surrounding the eggs). Previous work indicates that eggs can be fertilized up to 60 s from activation (which occurs once eggs come in contact with freshwater; Yamamoto 1961). We tested the length of the fertilization window by adding freshly activated sperm (to avoid the confounding effect of postejaculatory sperm aging) from the same male to four different experimentally split egg pools from the same female after 0, 15, 30, and 45 s from egg activation. We used 12 females and 12 males in total.

Experiment B: FRF Effect on the Fertilization Window. We tested whether the presence of FRF affects the egg fertilization window by comparing the fertilization rate in the presence and absence of FRF at two time points: 0 and 45 s from egg activation. For each female, eggs were collected, rinsed to remove the original FRF (for more details, see "Gametes and FRF Collection"), and split into four equal pools. FRF was then readded to two of these egg pools. Freshly activated sperm were added to the eggs 0 and 45 s after egg activation and the fertilization success was recorded. For this experiment, we obtained 20 experimental replicates (20 male-female pairs).

Experiment C: Multiple Paternity in the Presence or Absence of FRF. We tested whether the effect of FRF on the fertilization window provides more opportunities for postmating sexual selection by increasing the degree of multiple paternity. To do so, for each replicate we collected ejaculates from two males (labeled A and B) and the eggs and FRF from one female, so each replicate involved two males and one female, for a total of 15 replicates (2 males/1 female triplets). Once collected, the eggs were rinsed (as described below) to remove the FRF and split into two pools with the same number of eggs. In one of the two pools, the FRF was readded to the eggs. Freshly activated sperm from the first male (male A) were added immediately after egg activation (time 0), while freshly activated sperm from the second male (male B) were added after 30 s (time 30 s). We took care to use the same amount of sperm from the two competing males. Fin clips from the caudal fin of all the adults in the triplets were obtained after gamete collection and preserved in absolute ethanol until used for molecular analyses. Embryos were collected and preserved in absolute ethanol at 30 h postfertilization for paternity analysis.

Gametes and FRF Collection

The evening before the experiment, experimental fish were transferred into breeding tanks (1 L), where males and females remained separated by a transparent divider that allowed visual and olfactory contact but prevented physical interaction and spawning. Gametes were collected the next morning following Alavioon et al. (2017). In short, the fish were anesthetized in a solution of MS222 (tricaine methanesulfonate, 0.17 g/L; Sigma Aldrich), gently rinsed with water, and carefully dried in the abdominal and genital area (to prevent accidental activation of gametes by water). Each fish was then placed under a dissecting microscope for the collection of gametes. Males were gently squeezed to release the ejaculate, which was collected in a glass microcapillary, diluted in 40 μL of Hank's balanced salt solution (HBSS; Jing et al. 2009), and maintained on ice until used (within 1 h). Females were gently squeezed in the abdominal area to release eggs, along with the FRF, onto a glass slide. The FRF was carefully collected with a Drummond micropipette (see Poli et al. 2019) and maintained on ice until use. The eggs were then rinsed of the remaining fluid with a 0.5% solution of bovine serum albumin (pH 8), which allows maintaining eggs in an inactivated state for up to 2 h (Sakai 1997). Both eggs and FRF were always used within an hour of collection.

In Vitro Fertilization

The eggs of each female were divided into four (experiment B) or two (experiment C) pools (egg number range per pool of 30–60, with the same number of eggs used in each pool from the same female) and then activated with freshwater (FRF-absent treatment, hereafter referred to as the "no-FRF treatment") or with FRF plus freshwater (at a concentration of 1 : 10; FRF-present treatment, hereafter referred to as the "FRF treatment"). Sperm number was standardized by assessing sperm number with a LUNA automated cell counter and diluting each ejaculate accordingly with HBSS (Cattelan and Gasparini 2021). In all experiments, sperm were activated with freshwater (1 : 5 dilution) and added immediately to the appropriate egg pool. In experiment A, sperm were added at 0, 15, 30, or 45 s after egg activation; in experiment B, at 0 or 45 s after egg activation; and in experiment C, at 0 or 30 s after egg activation. After fertilization, eggs were incubated at 28°C and checked at 7 h postfertilization to assess fertilization success. The repeatability of the estimation of the fertilization success was confirmed in a separate experiment

using 10 male-female pairs, each with two replicates at 0 and 30 s.

Microsatellite and Parentage Analysis

Tissues for DNA analyses (the whole body of the embryos and fin clips from adults) were preserved in absolute ethanol until required. Genomic DNA was extracted using a protocol for the isolation of polymerase chain reaction (PCR)–ready genomic DNA from zebrafish tissues (Meeker et al. 2007). All individuals were genotyped at five microsatellite loci (GenBank accession numbers Z4830, Z20450, Z11496, Z9230, and Z1233) in multiplex PCRs performed in 15-μL reaction volumes following a cycling protocol with an initial denaturation step at 95°C for 10 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 35 s, and extension at 72°C for 50 s; and a final extension at 72°C for 30 min. PCR amplifications were performed on a SimpliAmp thermal cycler (Applied Biosystems). Amplified fragments were separated by electrophoresis on an ABI 3100 genetic analyzer (ABI PRISM, Applied Biosystems), using the GeneScan 500 LIZ (Applied Biosystems) as the size standard (https://www.bmr-genomics.it). Microsatellites were scored using the software Geneious (ver. 8.1.9; https://www.geneious.com), and paternity was assigned using Cervus (ver. 3.0; Kalinowski et al. 2007) with 95% strict confidence.

Statistical Analyses

All statistical analyses were conducted in R (ver. 3.6.3; R Core Team 2020). Repeatability was tested using the rptR package with proportion distribution, based on 1,000 permutations. Repeatability of fertilization success was high both at 0 s ($R = 0.024$ [95% confidence interval: 0.003–0.054], $P < .001$) and at 30 s ($R = 0.063$ [95% confidence interval: 0.021–0.098], $P < .001$).

To investigate the effect of FRF on the proportion of fertilized eggs (experiment B) and on the degree of multiple paternity (experiment C), we used a generalized linear mixed effect model (glmer function of the lme4 package), assuming a binomial error distribution. In the first model the number of fertilized and nonfertilized eggs was added as the dependent variable (using the cbind function), while in the second model the number of eggs sired by the second male (male B) and the number sired by the first male (male A) were added as the dependent variables (using the cbind function). In both models, treatment (presence/absence of FRF) was included as fixed factor and female ID was included as a random factor to account for the nonindependence of the data. No overdispersion was found in both models (assessed using the function testDispersion of the package DHARMa). The associated *P* value of the fixed factors was assessed using the Anova function (type II sum of squares) from the package car. Model assumptions were checked by inspection of residuals' distribution using the package DHARMa.

Averages are presented with their associated standard errors.

Results

Experiment A: Estimating the Zebrafish Fertilization Window

The average percentage of successfully fertilized eggs was 87.1% ± 1.4% at 0 s, 79.6% ± 1.5% at 15 s, 53.7% ± 4.4% at 30 s, and 19.7% ± 4% at 45 s (fig. 1).

Experiment B: FRF Effect on the Fertilization Window

At 0 s, the average percentage of successfully fertilized eggs was 83.7% ± 1.2% ($N = 20$) in the FRF treatment and 80.7% ± 1.2% ($N = 20$) in the no-FRF treatment. At 45 s, 25.1% ± 2.5% ($N = 20$) of the eggs were fertilized in the FRF treatment, while the percentage dropped to 16.5% ± 2.1% ($N = 20$) in the no-FRF treatment (fig. 2). The effect of the FRF on fertilization rate was nonsignificant at 0 s ($\chi^2_1 = 3.487$, $P = .062$) but was highly significant at 45 s ($\chi^2_1 = 23.557$, $P < .001$; fig. 2).

Experiment C: Multiple Paternity in the Presence or Absence of FRF

Overall, fertilization success obtained in the FRF treatment was significantly higher than in the no-FRF treatment (paired *t*-test: $t = 3.938$, df $= 14$, $P = .001$; within-pair mean difference of 9.1%). A mean of 51.3% ± 3.2% eggs were fertilized in the FRF treatment, and 42.2% ± 3.2% eggs were fertilized in the no-FRF treatment. We obtained a total of 541 embryos from 15 triplets (15 females and 30 males). We were able to assign paternity with 95% confidence using CERVUS to 507 (95%) embryos in total. The paternity was calculated for an average of 16.9 ± 1.3 embryos (range: 6–31) for each group. The second male's paternity (male B) ranged from 0% to 33% (mean: 13.3% ± 2.9%) in the no-FRF treatment and from 11% to 67% (mean: 41.8% ± 4.1%) in the FRF treatment, with an average within-pair difference of 27.7% ± 4%. There was a significant effect of FRF on the relative paternity of the second male ($\chi^2_1 = 41.49$, $P < .001$), with the second male (male B) siring more embryos when FRF was present (fig. 3).

Discussion

Overall, our combined results provide evidence that FRF prolongs the time available for egg fertilization (egg

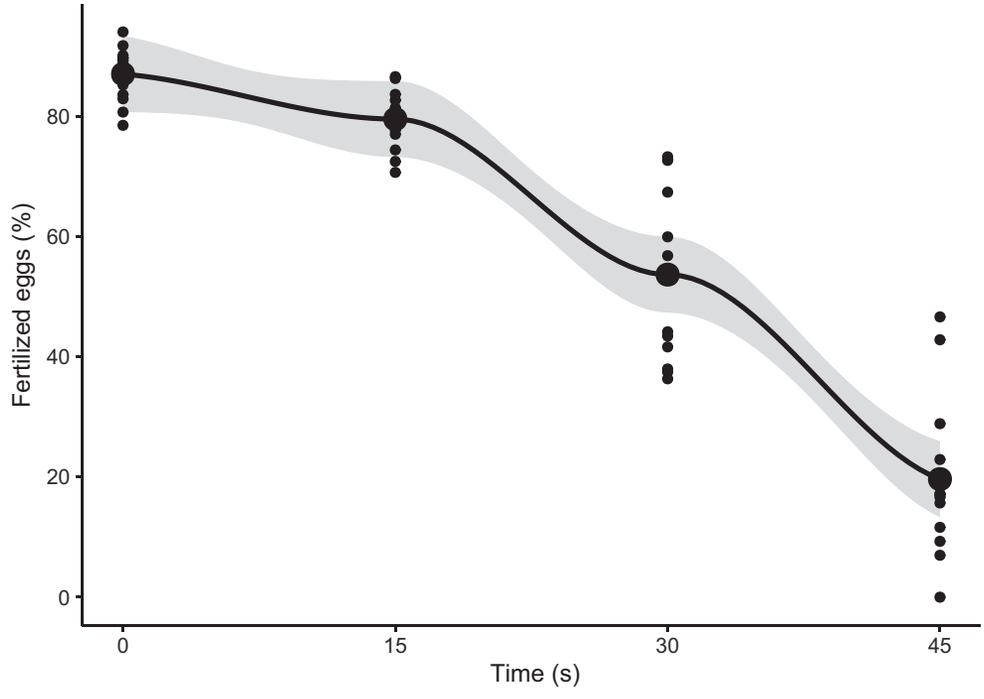

Figure 1: Experiment A—percentage of fertilized eggs over time obtained from in vitro fertilizations under standard conditions (i.e., with no female reproductive fluid manipulation). The individual data points (small circles; $N = 12$), means (big circles), and standard error of the mean (gray shading) are presented.

fertilization window) in the zebrafish. Moreover, we have demonstrated that the effect of FRF on eggs also increases the opportunities for postmating sexual selection and, as a consequence, the degree of multiple paternity of the offspring. This is the first experimental evidence of a link between the egg fertilization window and the opportunities for postmating sexual selection in external fertilizers.

We found that the fertilization window of zebrafish's eggs is characterized by a relatively short time frame (at 45 s after egg activation, only about 20% of the eggs are still fertilizable), with the majority of the eggs fertilized immediately after activation (eggs are activated when they come into contact with water). This result is in line with the timeframe of sperm longevity in this species, where the average duration of sperm motility is often less than 1 min (Wilson-Leedy et al. 2007; Poli et al. 2019). Interestingly, the presence of FRF can prolong both sperm longevity (Poli et al. 2019) and, as we demonstrated in this study, the egg fertilization window, thus increasing the possibilities of successful fertilization. The proportion of eggs fertilized toward the end of the fertilization window (45 s after egg activation) increased from 16% without FRF to 25% with FRF. This finding confirms the important role that FRF plays in maximizing fertilization success for in vitro fertilization protocols in fish husbandry (see, e.g., Turner and Montgomerie 2002; Lehnert et al. 2017). Mechanisms underlying this effect are to be found within the composition of the FRF; for example, in zebrafish the FRF contains protease inhibitors that prevent egg activation (Minin and Ozerova 2015) and that can also play a role in preserving egg fertilization ability. Furthermore, selection for longer-lived sperm was previously hypothesized for zebrafish (Poli et al. 2019), since this specific sperm phenotype has been shown to sire offspring with higher survival and adult fitness (Alavioon et al. 2019). Therefore, the extension of the egg fertilization window mediated by the FRF could represent a mechanism to reinforce this selection and ultimately increase offspring fitness, but specific studies are needed to test this idea.

Using molecular assignment of paternity, we found that in the presence of FRF there was a higher proportion of eggs fertilized by the second male. We estimated paternity on an average of 17 embryos per egg pool, and this was a consequence of our chosen experimental design; we acknowledge that this may have limited the accuracy of paternity estimation in those egg pools with fewer embryos genotyped. However, the use of a paired, balanced design mostly overcame this limitation, but aiming at genotyping more embryos in future studies of this type should be considered for a more precise estimation of paternity. Specifically, in presence of FRF the second male obtained an average proportion of paternity share that was 28%

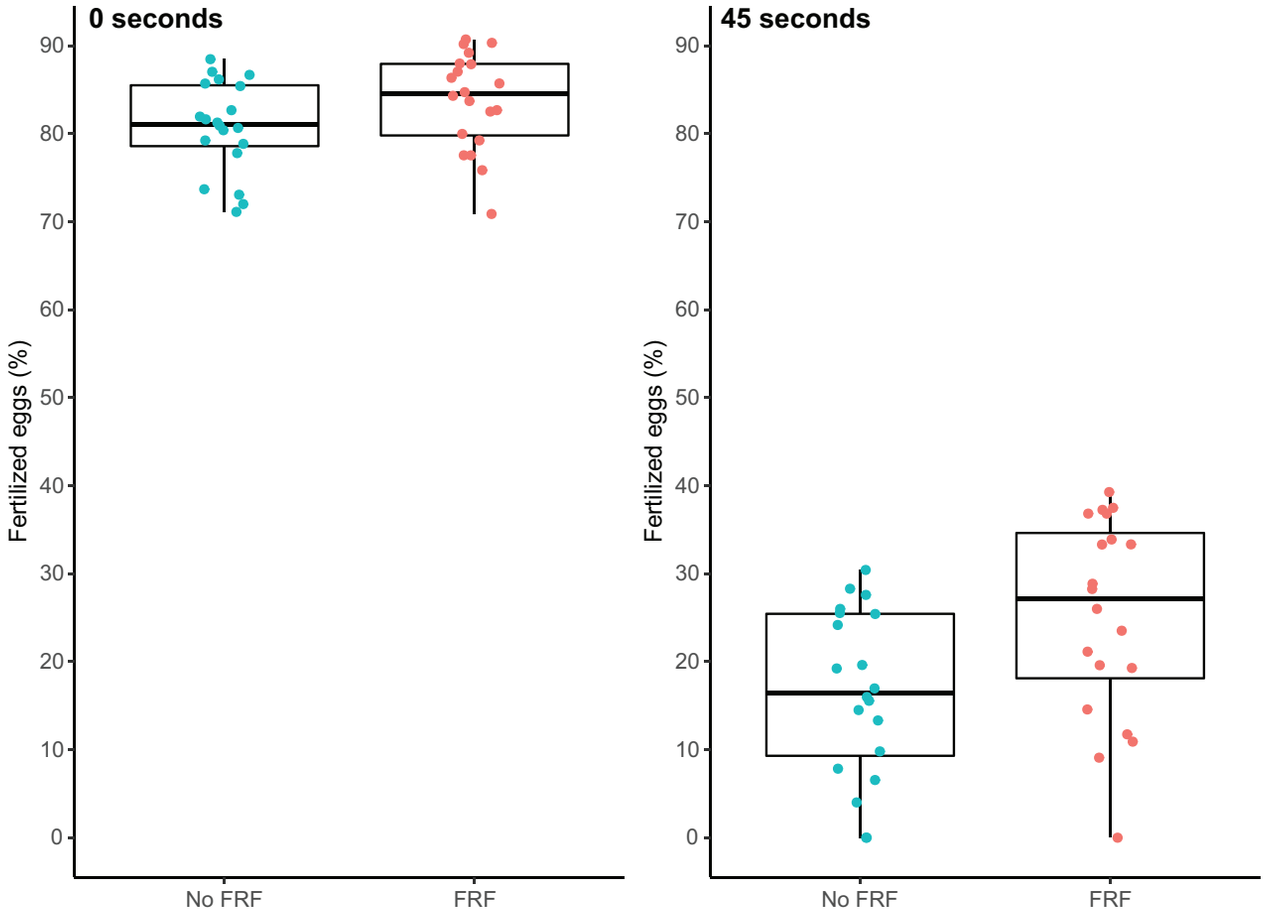

Figure 2: Experiment B—percentage of fertilized eggs obtained from in vitro fertilization in the absence (*left*) and presence (*right*) of female reproductive fluid (FRF) at 0 and 45 s. The presence of FRF significantly increased the percentage of fertilized eggs after 45 s from egg activation. The median (box midline), first (lower box line) and third (upper box line) quartiles, and range (whiskers) are presented. Each individual data point represents an experimental replicate ($N = 20$).

higher compared with in vitro fertilization in freshwater alone (41% in the presence of FRF, 14% in the absence of FRF). Our analyses revealed that the increased proportion of the second male is not merely due to the second male fertilizing some of the "remaining" eggs (those not fertilized by the first male) but to the second male actively competing for fertilization with the first male. Indeed, in the presence of FRF there was an overall higher fertilization rate (average difference: 9.1%; minimum difference: 1%; maximum difference: 31%), but the proportion of eggs fertilized by the second male was larger in magnitude than the cumulative fertilization (average difference: 27.7%; minimum difference: 4%; maximum difference: 58%), suggesting that the second male fertilized more than the eggs left unfertilized from the first male. Even if further studies are needed to confirm this point, it is therefore likely that the second male "steals" some eggs from the first male's fertilization potential.

Therefore, a longer fertilization window provides the opportunity for externally fertilizing females to mate polyandrously and thus to increase the occurrence and degree of multiple paternity. The evolutionary implications are various, as are the benefits associated with polyandry in the classical (premating) sense. First, there are fertility benefits for females. Indeed, females might benefit from exposing their eggs to multiple ejaculates to ensure an adequate sperm supply to fertilize all of the eggs and avoid sperm limitation, thus ultimately enhancing their fecundity (Jennions and Petrie 2000; Kraus et al. 2004; Snook 2014). This might represent insurance against the first male being sterile or releasing few or low-quality sperm. This may be particularly important in species where males become sperm depleted among successive mating events or when males modulate their sperm investment among different females (Birkhead and Moller 1998; Wedell et al. 2002).

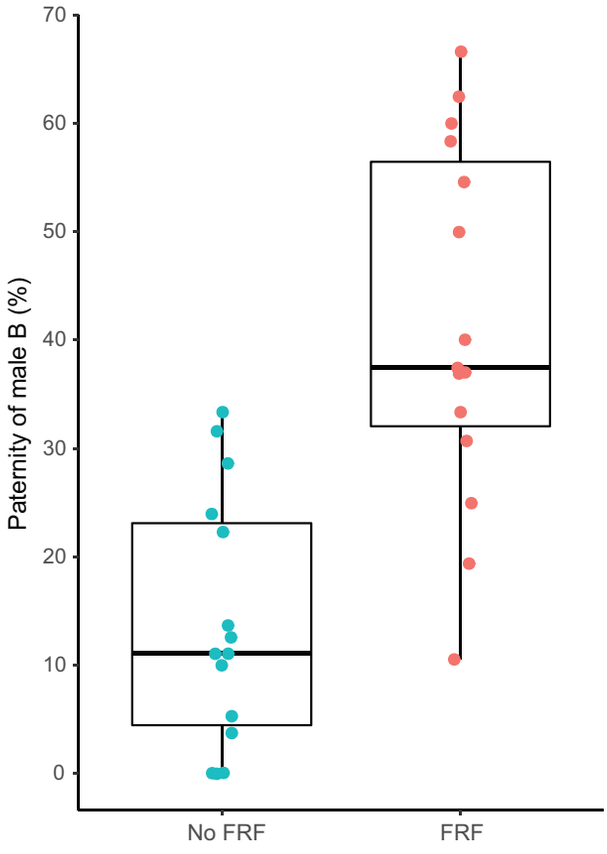

Figure 3: Experiment C—percentage of paternity obtained by the second male in in vitro fertilization trials in the absence (*left*) and presence (*right*) of female reproductive fluid (FRF). In the presence of FRF, the second male (whose sperm were added 30 s after the first male) fertilized significantly more eggs relative to when no FRF was present. The median (box midline), first (lower box line) and third (upper box line) quartiles, and range (whiskers) are presented. Each individual data point represents an experimental replicate ($N = 15$).

Moreover, the effect of FRF on multiple paternity has important implications for sexual selection, as it creates more opportunities for postmating sexual selection to act and to provide indirect benefits for females. Multiple paternity in the offspring could be favored by genetic bet hedging: the production of more genetically diverse offspring could be a strategy to increase the chances that some offspring will survive in heterogeneous environments characterized by variable selection on fitness-enhancing traits (Jennions and Petrie 2000). Alternatively, it may be a way for females to bias fertilization toward some specific males and thus exert cryptic female choice, for example, to bias fertilization toward unrelated or more compatible partners or more generally for preferred phenotypes (Eberhard 1996; Firman et al. 2017). This may be especially important when premating cues are not available or less reliable (Zeh and Zeh 1997; Birkhead and Pizzari 2002). In zebrafish, male premating competition plays a significant role in reproduction, with matings likely to be skewed toward the dominant male that is often able to exclude other males from the reproductive event rather than by females actively choosing their mates (Spence et al. 2008). However, despite this ability of the dominant male, reproduction in zebrafish is often characterized by multiple paternity shared between the dominant and the subordinate male or males (Watt et al. 2011), suggesting that postmating sexual selection plays an important role in the reproduction of this species. Our experimental design mimics this natural situation in which a female spawns in the presence of a male (likely the dominant) and a second male joins the pair slightly later (in our design, 30 s later); the presence of FRF provides the second male with the opportunity to compete for fertilization and the female with the possibility of exerting a postmating mate choice.

In conclusion, our findings corroborate and expand the role that FRF plays in postmating sexual selection, adding new information on how females of externally fertilizing species may affect these processes. It is precisely in these species, where females have limited control over males' competition for fertilization, that postmating mechanisms are expected to play a key role (Evans and Sherman 2013). Nonetheless, known mechanisms of cryptic female choice in external fertilizers are limited compared with internal fertilizers, and those mediated by FRF seem to be one of the best candidates (see Firman et al. 2017). However, little is known about how FRF can affect competitive fertilization (Gasparini et al. 2020), so the findings of this study expand the horizons of how FRF can affect the sexual selection processes by adding a new mechanism acting on the eggs rather than on the sperm, which creates novel opportunities for sperm competition and multiple paternity in external fertilizers.

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Statement of Authorship

L.P., F.P., M.B.R., and C.G. conceived the study. L.P. and C.G. designed the experiments. L.P. developed the methods, collected and analyzed the data, and wrote the original draft. A.G. contributed to the development of paternity methods. C.G. acquired the funding and participated

in data analysis. All authors reviewed and edited the draft and contributed to the final version of the manuscript.

Data and Code Availability

All data files are available from the Dryad Digital Repository (https://doi.org/10.5061/dryad.83bk3j9tr; Pinzoni et al. 2022).

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